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Muller, B

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DARWIN REVIEW

Generic signal-specific responses: cytokinin and context-dependent cellular responses

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Abstract

The phytohormone cytokinin triggers numerous and diverse responses during the plant life cycle via a two-component phosphorelay signalling system. Each step of the signalling cascade is supported by a gene family comprising several members. While functional redundancy is observed among family members, additional gene-specific functions encoded by *cis*-regulatory and coding sequence of individual family members have been described and contribute to specificity in signalling output. In addition, the cellular context of the signal-receiving cell affects the response triggered. Recent studies in *Arabidopsis* have demonstrated how expression of cytokinin signalling components predefines a spatiotemporal map of signalling sensitivity, which causes local signal amplification and attenuation. In summary, the specific interpretation of cytokinin signalling is affected by an orchestrated interplay of signalling genes and cellular context.

Key words: *Arabidopsis thaliana*, cytokinin signalling, development, pattern formation, plant hormones, signalling specificity.

Introduction

Plants and animals represent parallel realizations of multicellular development and display vastly different life cycles. They have independently evolved genes for pattern formation and cell–cell communication (Meyerowitz, 2002). In spite of the differences, common developmental strategies exist. For example, in contrast to the countless and diverse instances of cell-to-cell signalling, a surprisingly small repertoire of signal transduction systems is used. Research of the past two decades has revealed that the deployment of each transduction pathway is very flexible, and that a handful of core pathways are repeatedly implemented throughout metazoan development to account for innumerable cases of cell specification events (Davidson and Erwin, 2006). Similarly, in plants, signalling cascades triggered by phytohormones are used throughout the life cycle to affect cellular identities and responses (Santner *et al.*, 2009). Given the contrast between the few signalling systems and the innumerable cellular responses they trigger, the general question arises as to how fairly ubiquitous and generic signals can elicit specific and context-dependent cellular responses? Generally, specificity is achieved by regulating the localization, quantity, and quality of the signalling activity, and the status of the receiving cell. In this review, specificity and regulation of intracellular signalling will be discussed with regard to the cytokinin signalling circuitry; for discussions about how the availability and localization of active ligands is regulated, the interested reader is referred to recent reviews (Werner and Schmülling, 2009; Kudo *et al.*, 2010).

Cytokinins comprise a class of adenine-derived signalling molecules. The first cytokinin, Kinetin, was isolated as a breakdown product from autoclaved herring-sperm DNA, based on its cell-division promoting activity in cultivated tobacco tissue (Miller *et al.*, 1955; Amasino, 2005). Later, a number of additional effects provoked by cytokinins were described: stimulated leaf expansion and seed germination (Miller, 1956, 1958), delayed senescence in leaves (Richmond and Lang, 1957; Gan and Amasino, 1995), *de novo* organ formation from cultured tissue (Skoog and Miller, 1957), or release from apical dominance (Wickson and Thimann, 1958; Cline *et al.*, 1997), and more (Mizuno, 2005; To and Kieber, 2008; Jeon *et al.*, 2010; Perilli *et al.*, 2010).

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Cytokinins are perceived by a multistep phoshorelay system

The identification of the *CYTOKININ INDEPENDENT ONE (CKI1)* gene from *Arabidopsis thaliana* (Kakimoto, 1996) was the long-awaited breakthrough, which initiated the molecular elucidation of the cytokinin signal transduction pathway in the following years. Its overexpression caused the typical cytokinin responses in tissue culture (Kakimoto, 1996) and *in planta* (Hwang and Sheen, 2001). *CKI* encodes for a hybrid kinase, suggesting it functions in a phosphorelay system. Phosphorelay, or two-component signalling systems, are prevalent in bacteria (West and Stock, 2001). In the simplest form, they consist of two conserved proteins: a histidine protein kinase and a response regulator protein that are phosphorylated at conserved His and Asp residues, respectively. Phosphotransfer from the histidine kinase to the response regulator results in activation of the latter (Fig. 1A). More complex versions of this two-component phosphotransfer involve multiple phosphotransfer steps, and often more than two proteins (Fig. 1B). Further evidence supporting the use of a phosphorelay system for cytokinin signalling came when additional genes with conserved His- and Asp-containing domains were identified, such as response regulators (RR), and histidine phosphotransfer proteins (HPt) (Mok and Mok, 2001). A forward genetic screen using tissue culture assays led to the identification of the *cytokinin response 1-1 (cre1-1)* mutation, allelic to the previously characterized *woodenleg (wol)* mutation (Mähönen *et al.*, 2000; Inoue *et al.*, 2001). The same gene was independently isolated and named as *ARABIDOPSIS HISTIDINE KINASE 4 (AHK4)* (Ueguchi *et al.*, 2001). The *CRE1/WOL/AHK4* gene encodes for a true cytokinin receptor that, unlike CKI1, could bind and respond to cytokinins (Suzuki *et al.*, 2001; Yamada *et al.*, 2001). The completion of the *Arabidopsis* genome sequence allowed researchers systematically to compile the potential signalling components, based on the characteristic signatures of domains implicated in phosphorelay signalling (Fig. 2; Hwang *et al.*, 2002). Functional reconstitution of the pathway in an *Arabidopsis* cellular system established the core logic of the pathway (Fig. 1B, Hwang and Sheen, 2001). Pathway activation is initiated by autophosphorylation at a conserved His residue of the receptors, which is subsequently carried-over to a conserved Asp of the receiver domain. The phosphoryl group is then transferred to the functional *Arabidopsis* histidine phosphotransfer proteins (AHP) proteins (Imamura *et al.*, 1999; Suzuki *et al.*, 2000). AHP proteins can form dimers and shuttle between nucleus and cytosol in a cytokinin-independent manner (Punwani *et al.*, 2010). From the AHPs, the phosphate gets passed-over to the nuclear-localized response regulators. Type-B *Arabidopsis* response regulators (type-B ARR) act as transcriptional activators, while type-A *Arabidopsis* response regulators (type-A ARR) negatively interfere with pathway activity, most likely by competing with type-B ARRs for access to the AHPs, and by acting as phosphate sinks (To *et al.*, 2007). Transcription of type-A *ARR* is directly induced by the activated B-type ARRs (D'Agostino *et al.*, 2000), which establishes a negative feedback loop to the pathway (Hwang and Sheen, 2001). Structurally and functionally, the type-C ARRs resemble type-A ARRs, as they contain a conserved receiver domain and their ectopic expression represses cytokinin output strongly. However, unlike type-A *ARR*s, their expression does not depend on cytokinin. No aberrant phenotypes have been observed with loss-of function mutants under normal growth conditions, however, since *ARR22* expression was found to be induced by wounding, their functions may become relevant upon conditions of mechanical stress (Kiba *et al.*, 2004; Gattolin *et al.*, 2006; Horák *et al.*, 2008).

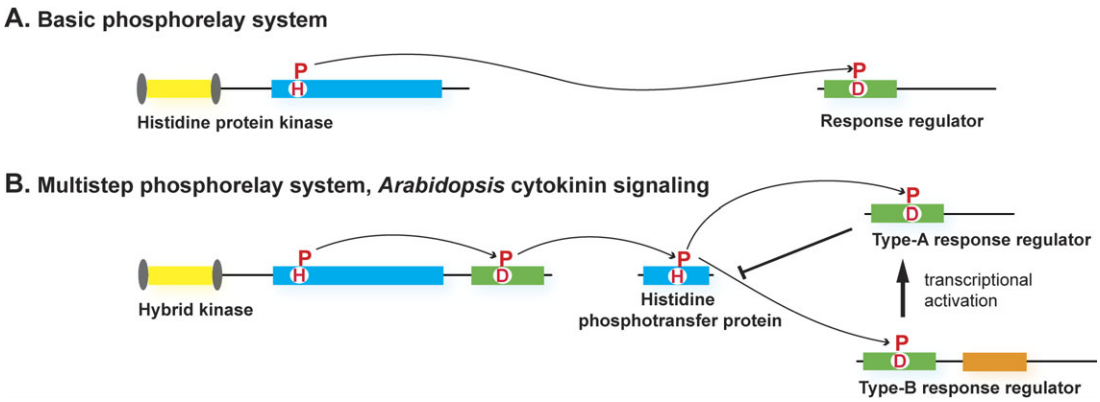

Fig. 1. Schematic representation of the two-component and the multistep phosphorelay signalling systems. (A) The basic phosphorelay system uses a single phosphoryl transfer event between a His protein kinase and its cognate response regulator. (B) The multistep His-to-Asp phosphorelay system, in which a His-containing phosphotransfer protein (HPt) serves as a phosphoryl acceptor and donor between the hybrid protein kinase (HK) and the response regulators (RR). The activation of the type-B RRs results in transcriptional activation. Type-A RRs attenuate signalling activity. Transcriptional activation of repressive type-A *RR*s establishes a negative feedback loop to the pathway.

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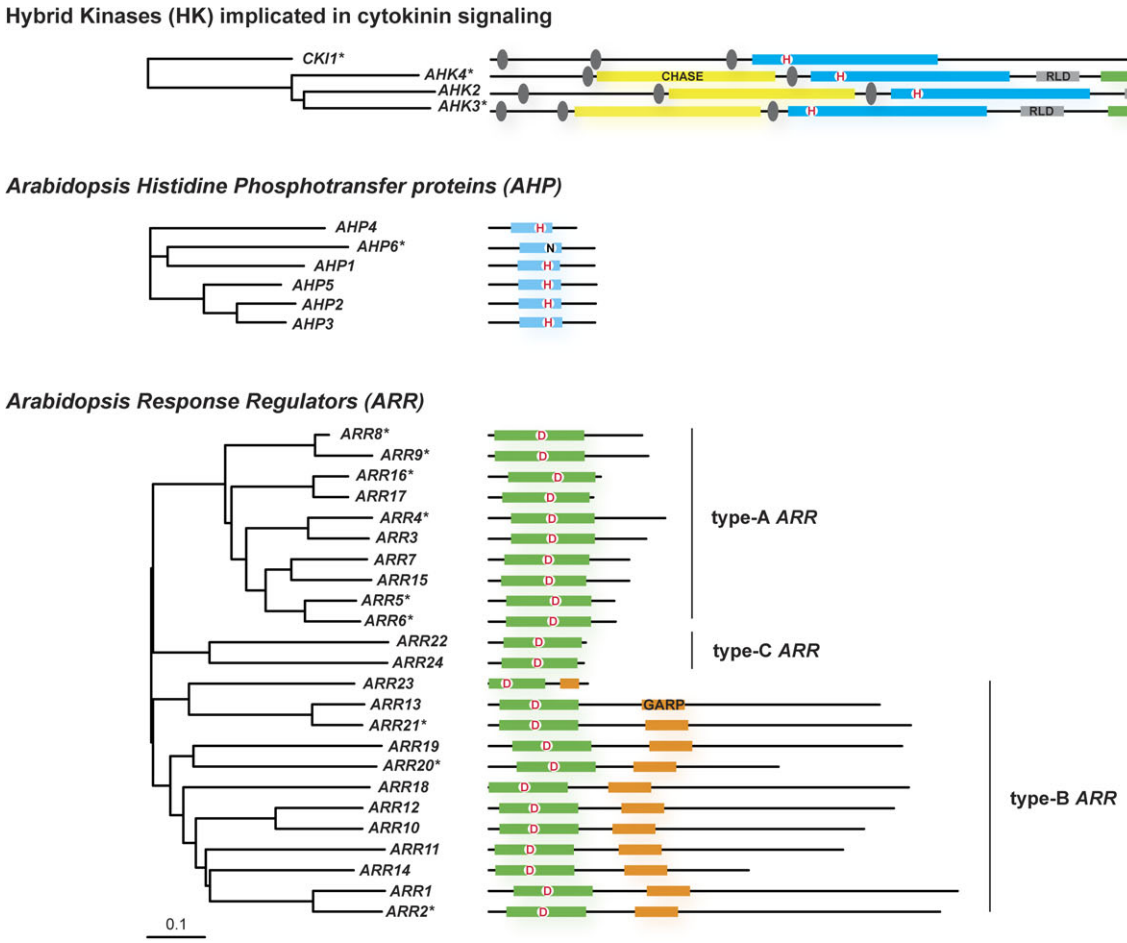

Fig. 2. Compilation of cytokinin signalling components from *Arabidopsis*, and their phylogenetic relationship. On the left, the phylogenetic relationship of *Arabidopsis* family members is shown for the HKs with a well-defined role in cytokinin signalling, for the AHPs, and the ARRs. Alignments were created using the MAFFT-E-INS-i algorithm (Katoh and Toh, 2008), based on full-length protein sequences retrieved from TAIR (Swarbreck *et al.*, 2008). Unrooted Neighbor–Joining trees (Saitou and Nei, 1987) were calculated using a BLOSUM62 matrix. The scale bar represents 0.1 amino acid substitutions per site. On the right, the proteins are indicated as black lines. Conserved domains are indicated as boxes. The cytokinin-binding CHASE domains are drawn as yellow boxes, transmembrane segments as grey ovals. Receptor-like domains (RLD) as a grey box. Kinase/transmitter (in HKs) and transmitter domains (in AHPs) with the conserved His residue that gets phosphorylated are depicted as blue boxes; receiver domains, with the conserved Asp residue (D), are indicated by a green box. The DNA-binding GARP domain of the type-B ARRs is indicated by an orange box. The GARP domain of ARR23 is truncated. Asterisks denote genes products with a documented specific function within cytokinin signalling, as discussed in the text.

Functional studies reveal redundant and gene-specific functions of family members

For each step in the signalling cascade, multiple genes are encoded in the *Arabidopsis* genome (Fig. 2; Table 1). The question arises as to which degree the expansion of each gene family reflects functional diversification? On the one hand, functional studies *in planta* and in heterologous systems supported the core function of different family members in the signalling circuitry; on the other hand, gene-specific functions emerged as well. Sequence comparisons within protein families reveal stretches of poor conservation (Fig. 2). For example, beyond their conserved domains, both type-A and type-B ARRs harbour stretches of sequences that exhibit little similarity within the protein family. Such variable parts can be expected to mediate gene-specific functions. Furthermore, the analysis of the transcription profiles of cytokinin signalling components *in planta* uncovered complex patterns (Ferreira and Kieber, 2005), which reflects diversity in expression. Thus, gene-specific functions appear to depend both on protein sequence and expression patterns.

In general, loss-of-function mutations in single genes resulted in only subtle phenotypes, revealing the functional redundancy within gene families (Table 1). An exception is the hybrid kinase *CKI1*; it's loss caused female gametophyte lethality (Pischke *et al.*, 2002; Hejátko *et al.*, 2003; Deng *et al.*, 2010). To obtain stronger phenotypes, plants were generated that harbour loss-of-function mutations in several to all genes belonging to a family, comprising the true receptors (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004;

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Table 1. List of mutants affecting cytokinin signalling genes, including higher-order mutants

Gene abbreviation	Mutants	Molecular characterization	Phenotypes	References
Hybrid kinases				
CKI1	*cki1-1,1-2,1-3,1-4*	Gain-of-of function, T-DNA activation tagging mutants, insertions in promoter of *CKI1*	Ectopic cytokinin activities, resulting in absence of roots and normal flowers, and sterility	Kakimoto, 1996
	cki1-6	Loss-of-function, T-DNA insertion in exon 3	Female gametophyte lethality	Pischke *et al.*, 2002
	ckil-1	Loss-of-function, En-1 transposon insertion in exon 6	Female gametophyte lethality	Hejátko *et al.*, 2003
	CKI1(RNAi) 5-3	RNA-interference transgene, causing reduction of endogenous *CKI1* mRNA levels	Vascular defects	Hejátko *et al.*, 2009
	cki1-8	Loss- and gain-of function, estradiol-inducible activation tagging T-DNA insertion in promoter of *CKI1*	Ectopic cytokinin activities upon estradiol applications, female gametophyte lethality without estradiol	Deng *et al.*, 2010
AHK4	*wol*	Recessive, dose-dependent dominant negative allele, missense mutation T278I	Determinate root growth phenotype, resulting from a reduced number of cell divisions in the procambium and abnormal differentiation of all vascular cells into protoxylem	Mähönen *et al.*, 2006*b*, 2000
	cre1-1	Loss-of function, missense mutation G437D	None under normal conditions, reduced cytokinin-dependent shoot formation in tissue culture, cytokinin-insensitive root growth	Inoue *et al.*, 2001
	crei1-2	Loss-of function, T-DNA insertion in exon 2	Increased primary root length	Inoue *et al.*, 2001; Riefler *et al.*, 2006
	ahk4-1	Hypomorphic, T-DNA insertion in intron 9	None under normal conditions, cytokinin-insensitive root growth	Nishimura *et al.*, 2004
	cre1-12	Loss-of-function, T-DNA insertion in intron 2	None under normal conditions, reduced relative WUS transcript levels in SAM upon ectopic cytokinin application	Higuchi *et al.*, 2004; Gordon *et al.*, 2009
AHK3	*ahk3-1*	Loss-of-function, T-DNA insertion in exon 9	None under normal conditions	Nishimura *et al.*, 2004
	ahk3-3	Hypomorphic, T-DNA insertion in intron 6	Increased root meristem cell number and root growth	Higuchi *et al.*, 2004; Dello Ioio *et al.*, 2007
	ahk3-7	Loss-of-function, T-DNA insertion in intron 1	Reduced rosette diameter, reduced chlorophyll content, increased root meristem cell number and root growth	Riefler *et al.*, 2006; Dello Ioio *et al.*, 2007
	ore12-1	Gain-of-function, missense mutation P243S	Delayed leaf senescence	Kim *et al.*, 2006
AHK2	*ahk2-1*	Loss-of-function, T-DNA insertion in exon 7	None under normal conditions	Nishimura *et al.*, 2004

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Table 1. *Continued*

Gene abbreviation	Mutants	Molecular characterization	Phenotypes	References
	ahk2-2	Loss-of-function, T-DNA insertion in exon 5	None under normal conditions, reduced relative WUS transcript levels in SAM upon ectopic cytokinin application	Higuchi *et al.*, 2004; Gordon *et al.*, 2009
	ahk2-5	Loss-of-function, T-DNA insertion in exon 4	None under normal conditions	Riefler *et al.*, 2006
AHK2, AHK3	*ahk2-1 ahk3-1*		Semi-dwarf phenotype in aerial part	Nishimura *et al.*, 2004
	ahk2-2 ahk3-3		Smaller leaves, shorter stems than wild type	Higuchi *et al.*, 2004
	ahk2-5 ahk3-7		Markedly reduced rosette size, reduced final height, strongly reduced chlorophyll content, strongly enhanced root branching and primary root growth	Riefler *et al.*, 2006
AHK2, AHK4	*ahk2-1 ahk4-1*		None under normal conditions	Nishimura *et al.*, 2004
	ahk2-2 cre1-12		None under normal conditions	Higuchi *et al.*, 2004
	ahk2-5 cre1-2		Increased lateral root formation	Riefler *et al.*, 2006
AHK3, AHK4	*ahk3-1 ahk4-1*		None under normal conditions	Nishimura *et al.*, 2004
	ahk3-3 cre1-12		None under normal conditions	Higuchi *et al.*, 2004
	ahk3-7 cre1-2		Increased lateral root formation	Riefler *et al.*, 2006
AHK2, AHK3, AHK4	*ahk2-1 ahk3-1 ahk4-1*		Strongly reduced size due to fewer and smaller cells, sterile, strongly reduced SAM	Nishimura *et al.*, 2004
	ahk2-2 ahk3-3 cre1-12		Very slow root and shoot growth, decreased leaf number, sterile, strongly reduced SAM	Higuchi *et al.*, 2004
	ahk2-5 ahk3-7 cre1-2		Miniature plants, strongly reduced fertility, retarded flowering, strongly reduced chlorophyll content, increased seed size (maternal/endospermal effect),	Riefler *et al.*, 2006
Histidine phosphotransfer proteins				
AHP1	*ahp1*	Loss-of-function, T-DNA insertion in intron 1	None	Hutchison *et al.*, 2006
AHP2	*ahp2-1*	Hypomorphic, T-DNA insertion in intron 3	None	Hutchison *et al.*, 2006
	ahp2-2	Loss-of-function, T-DNA insertion in exon 4	None	Deng *et al.*, 2010
AHP3	*ahp3*	Loss-of-function, T-DNA insertion in exon 1	None	Hutchison *et al.*, 2006
AHP4	*ahp4*	Hypomorphic, T-DNA insertion in intron 4	None	Hutchison *et al.*, 2006
AHP5	*ahp5-1*	Loss-of-function, T-DNA insertion in exon 3	None	Hutchison *et al.*, 2006

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Table 1. *Continued*

Gene abbreviation	Mutants	Molecular characterization	Phenotypes	References
	ahp5-2	Loss-of-function, T-DNA insertion in intron 4	None	Hutchison *et al.*, 2006
AHP1, AHP2	*ahp1 ahp2*		None	Hutchison *et al.*, 2006
AHP1, AHP3	*ahp1 ahp3*		None	Hutchison *et al.*, 2006
AHP2, AHP3	*ahp2 ahp3*		None under normal conditions, slightly reduced sensitivity to exogenous cytokinins	Hutchison *et al.*, 2006
AHP2, AHP4	*ahp2 ahp4*		None	Hutchison *et al.*, 2006
AHP2, AHP5	*ahp2 ahp5-2*		None	Hutchison *et al.*, 2006
AHP3, AHP4	*ahp3 ahp4*		None	Hutchison *et al.*, 2006
AHP3, AHP5	*ahp3 ahp5-2*		None	Hutchison *et al.*, 2006
AHP4, AHP5	*ahp4 ahp5-2*		None	Hutchison *et al.*, 2006
AHP1, AHP5	*ahp1 ahp5-1*		None	Hutchison *et al.*, 2006
AHP1, AHP2, AHP3	*ahp1 ahp2 ahp3*		None under normal conditions, reduced sensitivity to exogenous cytokinins	Hutchison *et al.*, 2006
AHP2, AHP3, AHP4	*ahp2 ahp3 ahp4*		None	Hutchison *et al.*, 2006
AHP2 AHP3, AHP5	*ahp2 ahp3 ahp5-2*		Short, narrow primary root	Hutchison *et al.*, 2006
AHP1 AHP2, AHP3 AHP4	*ahp1 ahp2 ahp3 ahp4*		None under normal conditions, reduced sensitivity to exogenous cytokinins	Hutchison *et al.*, 2006
AHP2, AHP3, AHP4, AHP5	*ahp2-1 ahp3 ahp5-2*		short, narrow primary root	Hutchison *et al.*, 2006
AHP2, AHP3, AHP5	*ahp2-1 ahp3 ahp4 ahp5-1*		Short, narrow primary root	Hutchison *et al.*, 2006
AHP1, AHP2, AHP3, AHP4, AHP5	*ahp1 ahp2-1 ahp3 ahp5-2*		Short, narrow primary root, impaired vascular development, delayed flowering, overall small size	Hutchison *et al.*, 2006; Deng *et al.*, 2010
AHP1, AHP2, AHP3, AHP4, AHP5	*ahp1 ahp2-2 ahp3 ahp4 ahp5-2*		Gametophytic lethality, rare escapers seedling lethal	Deng *et al.*, 2010
AHP6	*ahp6-1*	Nonsense mutation in first exon	Defects in vasculature: impaired protoxylem differentiation	Mähönen *et al.*, 2006*a*
Type-A response regulators				
ARR3	*arr3*	Loss-of-function, T-DNA insertion in 3'-UTR	None	To *et al.*, 2004; Salome *et al.*, 2006
ARR4	*arr4*	Hypomorphic, T-DNA insertion in exon 5	Mildly elongated petioles	To *et al.*, 2004; Salome *et al.*, 2006
ARR5	*arr5*	Hypomorphic, T-DNA insertion in exon 4	Reduced rosette size	To *et al.*, 2004; Salome *et al.*, 2006
ARR6	*arr6*	Hypomorphic, T-DNA insertion in intron 4	None	To *et al.*, 2004; Salome *et al.*, 2006
ARR7	*arr7-1*	Loss-of-function, T-DNA insertion in exon 3	None	Leibfried *et al.*, 2005
	ARR7(RNAi)	EtOH-inducible RNA interference transgene	None	Müller and Sheen, 2008
ARR8	*arr8*	Loss-of-function, T-DNA insertion in exon 1	Slightly reduced lateral root formation	To *et al.*, 2004; Salome *et al.*, 2006
ARR9	*arr9*	Hypomorphic, T-DNA insertion in intron 4	Slightly reduced lateral root formation	To *et al.*, 2004; Salome *et al.*, 2006
ARR15	*arr15-1*	Loss-of-function, T-DNA insertion in exon 5	None	Leibfried *et al.*, 2005
	arr15-2	Loss-of-function, T-DNA insertion in exon 1	None	Müller and Sheen, 2008

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Table 1. *Continued*

Gene abbreviation	Mutants	Molecular characterization	Phenotypes	References
ARR16				
ARR17				
ARR3, ARR4	*arr3 arr4*		Lengthen period of circadian clock, elongated petioles, increased sensitivity to exogenous cytokinins	To *et al.*, 2004; Salome *et al.*, 2006
ARR4, ARR5	*arr4 arr5*		Like *arr5* parent, increased sensitivity to exogenous cytokinins	To *et al.*, 2004; Salome *et al.*, 2006
ARR5, ARR6	*arr5 arr6*		None	To *et al.*, 2004; Salome *et al.*, 2006
ARR4, ARR6	*arr4 arr6*		Like *arr4* parent	To *et al.*, 2004; Salome *et al.*, 2006
ARR8, ARR9	*arr8 arr9*		Reduced lateral root formation, increased sensitivity to exogenous cytokinins	To *et al.*, 2004; Salome *et al.*, 2006
ARR7, ARR15	*arr7 arr15*		Female gametophyte lethality	Leibfried *et al.*, 2005
	ARR7(RNAi) arr15		Defects in embryonic root meristem, defects in phyllotaxis	Zhao *et al.*, 2010; Müller and Sheen, 2008
	AlcA::AM7/15	EtOH-inducible microRNA	Defects in phyllotaxis	Zhao *et al.*, 2010
ARR5, ARR6, ARR8, ARR9	*arr5 arr6 arr8 arr9*		None under normal conditions	To *et al.*, 2004; Salome *et al.*, 2006
ARR3, ARR4, ARR5, ARR6	*arr3 arr4 arr5 arr6*		Significantly elongated petioles, increased sensitivity to exogenous cytokinins, higher chlorophyll content	To *et al.*, 2004; Salome *et al.*, 2006
ARR3, ARR4, ARR8, ARR9	*arr3 arr4 arr8 arr9*		Markedly increased sensitivity to exogenous cytokinins	To *et al.*, 2004; Salome *et al.*, 2006
ARR3, ARR4, ARR5, ARR6, ARR8, ARR9	*arr3 arr4 arr5 arr6 arr8 arr9*		Elongated petioles, hypersensitive to exogenous cytokinins	To *et al.*, 2004; Salome *et al.*, 2006
ARR3, ARR4, ARR5, ARR6, ARR7, ARR8, ARR9	*arr3 arr4 arr5 arr6 arr7 arr8 arr9*		Defects in phyllotaxis	Leibfried *et al.*, 2005
Type-B response regulators				
ARR1	*arr1-1*	Loss-of-function, T-DNA insertion in intron 1	Slightly enlarged primary root	Sakai *et al.*, 2001
	arr1-3	Loss-of-function, T-DNA insertion in exon 5	None under normal conditions	Mason *et al.*, 2005; Argyros *et al.*, 2008
	arr1-4	Loss-of-function, T-DNA insertion in exon 3	None under normal conditions	Ishida *et al.*, 2008
ARR2	*arr2-4*	Loss-of-function, T-DNA insertion in exon 4	None under normal conditions, reduced pathogen resistance	Mason *et al.*, 2005; Choi *et al.*, 2010
	arr2	Loss-of-function, DS insertion in exon 1	Retarded growth, late flowering, reduced ethylene sensitivity	Hass *et al.*, 2004
ARR10	*arr10-2*	Loss-of-function, T-DNA insertion in intron 5	None under normal conditions	Mason *et al.*, 2005
	arr10-5	Loss-of-function, T-DNA insertion in exon 5	None under normal conditions	Ishida *et al.*, 2008; Argyros *et al.*, 2008
ARR11	*arr11-2*	Loss-of-function, T-DNA insertion exon 2	None under normal conditions	Mason *et al.*, 2005
ARR12	*arr12-1*	Loss-of-function, T-DNA insertion in exon 3	None under normal conditions	Mason *et al.*, 2005; Argyros *et al.*, 2008

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Table 1. *Continued*

Gene abbreviation	Mutants	Molecular characterization	Phenotypes	References
ARR14	*arr14-1*	Loss-of-function, T-DNA insertion in exon 2	None under normal conditions	Ishida *et al.*, 2008
ARR18	*arr18-2*	Hypomorphic, T-DNA insertion in 5'-UTR	None under normal conditions	Mason *et al.*, 2005
ARR19				
ARR20				
ARR21		Loss-of-function, dSPm insertion in intron 3	None	Horák *et al.*, 2003
ARR1, ARR10	*arr1-1 arr10-2*		None under normal conditions, reduced sensitivity to exogenous cytokinins	Mason *et al.*, 2005
ARR1, ARR11	*arr1-1 arr11-2*		None under normal conditions, reduced sensitivity to exogenous cytokinins	Mason *et al.*, 2005
ARR1, ARR18	*arr1-3 arr18-2*		None under normal conditions, reduced sensitivity to exogenous cytokinins	Mason *et al.*, 2005
ARR10, ARR11	*arr10-2 arr11-2*		None under normal conditions, reduced sensitivity to exogenous cytokinins	Mason *et al.*, 2005
ARR11, ARR18	*arr11-2 arr18-2*		None under normal conditions, reduced sensitivity to exogenous cytokinins	Mason *et al.*, 2005
ARR1, ARR10, ARR11	*arr1-1 arr10-2*		None under normal conditions, reduced sensitivity to exogenous cytokinins	Mason *et al.*, 2005
ARR1, ARR10, ARR12	*arr1-3 arr10-2 arr12-1*		None under normal conditions, reduced sensitivity to exogenous cytokinins	Mason *et al.*, 2005
	arr1-4 arr10-5 arr12-1		Severe growth defect, resistance to exogenous cytokinins, vascular defects, reduced SAM	Ishida *et al.*, 2008
	arr1-3 arr10-5 arr12-1		Short narrow primary root, altered vascular development, reduced rosette size, reduced chlorophyll content, impaired response to exogenous cytokinins, increased seed size	Argyros *et al.*, 2008
Type-C response regulators				
ARR22	*arr22*	Loss-of-function, T-DNA insertion in exon 1	None	Gattolin *et al.*, 2006
	arr22-2, arr22-3	Loss-of-function, T-DNA insertion in intron 2	None	Horák *et al.*, 2008
ARR24	*arr24*	Loss-of-function, T-DNA insertions in exon	None	Gattolin *et al.*, 2006
ARR22, ARR24	*arr22 arr24*		None	Gattolin *et al.*, 2006

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Riefler *et al.*, 2006), *AHP*s (Hutchison *et al.*, 2006; Deng *et al.*, 2010), type-A *ARR*s (Hutchison *et al.*, 2006; Salome *et al.*, 2006; To *et al.*, 2007), and type-B *ARR*s (Mason *et al.*, 2005; Argyros *et al.*, 2008; Ishida *et al.*, 2008). The results from these studies confirmed the extensive redundancy within each gene family: phenotype severity and insensitivity to cytokinin increased with the number of affected family members. In addition, gene-specific functions were uncovered (Table 1). In principle, these can be explained by the differential expression patterns, or by the specific protein functions of the genes involved. In the first scenario, loss of the family members with the highest expression levels in a given tissue is expected to result in the strongest phenotype. For example, *AHK4*'s functional contribution for the root system (Ueguchi *et al.*, 2001; Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006) reflects its relatively higher overall expression levels in root tissue (Ueguchi *et al.*, 2001; Higuchi *et al.*, 2004). In the transition zone of the primary root meristem however, *AHK3* expression (Nishimura *et al.*, 2004) and function prevails (Dello Ioio *et al.*, 2007). In the shoot apical meristem (SAM), *AHK2* and *AHK4,* but not *AHK3,* mediate cytokinin-dependent *WUSCHEL (WUS)* expression (Gordon *et al.*, 2009), which correlates with the low abundance of *AHK3* in the SAM (Nishimura *et al.*, 2004). Similarly, the strong phenotypes of a plant defective in three of the 11 type-B *ARR*s, *ARR1, ARR10*, and *ARR12* (Mason *et al.*, 2005; Yokoyama *et al.*, 2007; Ishida *et al.*, 2008) correlated with their relatively high and widespread expression (Mason *et al.*, 2004). Similarly, some of the type-A *ARR* mutant combinations exhibited specific phenotypes, which are consistent with the expression patterns of the affected genes. For example, *ARR8* and *ARR9* are expressed strongly throughout the root, and disrupting their loci affects lateral root number in seedlings but does not affect shoot development (To *et al.*, 2004). Disruption of *ARR7* and *ARR15* functions interferes with embryonic root meristem establishment in the early embryo (Müller and Sheen, 2008) and causes disturbed phyllotaxis in the SAM (Zhao *et al.*, 2010), in agreement with their unique expression patterns in these tissues. The reported female gametophyte lethality of an *arr7 arr15* double mutation (Leibfried *et al.*, 2005) could also reflect the expression patterns of *ARR7* and *ARR15* during this phase of development. The gene pairs *ARR8*/*ARR9* and *ARR7*/*ARR15* represent closely related sister genes (Fig. 2) and arose from the most recent *Arabidopsis* genome duplication (Vision *et al.*, 2000). Commonly, such sister genes share more redundant functions compared with more divergent pairs (Lynch and Conery, 2000). By analogy, analysing the missing double-mutant combinations of the *ARR* gene pairs *ARR16*/*ARR17*, *ARR19*/*ARR20,* or *ARR13*/*ARR21* (Fig. 2) could reveal interesting phenotypes. However, as an exception to the rule, the closely related type-B *ARR1* and *ARR2* genes (Fig. 2) share few functions (Hass *et al.*, 2004; Mason *et al.*, 2005; Choi *et al.*, 2010), despite their overlapping expression patterns (Mason *et al.*, 2004). Recently, the unique ability of ARR2 to confer cytokinin-mediated pathogen resistance has been mapped to the variable C-terminal domain beyond the conserved DNA-binding domain (Fig. 2). Via this portion of the protein, the transcription factor ARR2 is recruited by TGA3 to the *cis*-elements of the *PR1* promoter, leading to its transcriptional activation. Under the same conditions, ARR1 did not interact with TGA3 (Choi *et al.*, 2010). Gene-specific functions depending on the distinct biochemical functions of the affected genes were also described for other signalling components (see asterisks in Fig. 2). Similar to *AHK2, AHK3*, and *AHK4*, *CKI1* codes for a hybrid kinase, which activates cytokinin signalling. However, it lacks the CHASE (cyclase/His kinase-associated sensing extracellular) domain involved in cytokinin binding (Fig. 2; Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001). Indeed, *cki* plants still respond to exogenously applied cytokinins similar to the wild-type plants, supporting a specific function of *CKI1*, which activates signalling independent of cytokinins (Hejátko *et al.*, 2009; Deng *et al.*, 2010). Characterization of the *wol* allele of *AHK4* revealed that phosphotransfer is bi-directional. Compared with wild-type AHK4, the *WOL* gene product has an amino acid change in the putative extracellular, cytokinin-binding region and lacks cytokinin binding activity, which reveals the inherent phosphatase activity of AHK4 that normally depletes the circuitry of phosphoryl groups in the absence of ligands (Mähönen *et al.*, 2006*b*). AHK2 and AHK3 appear to lack a similar activity. *AHK3*, in turn, has been shown to exhibit unique functions in mediating cytokinin's function in leaf senescence (Kim *et al.*, 2006; Riefler *et al.*, 2006). Neither mutants of the other receptors, *AHK2* and *AHK4*, nor their overexpression affected leaf senescence (Kim *et al.*, 2006). Within the family of AHPs, AHP6 stands out by lacking a conserved His residue in its receiver domain (Fig. 2), which renders it unable to accept an activating phosphoryl group (Mähönen *et al.*, 2006*b*). Hence, it was named pseudo *AHP*. *ahp6* mutant plants show defects in protoxylem differentiation, reflecting an increase in cytokinin signalling levels. AHP6 negatively interferes with pathway activity, most likely by competing with AHP1–5 for interaction with the activated receptors (Mähönen *et al.*, 2006*a*).

More gene-specific protein functions were uncovered by overexpression studies done *in planta* or heterologous systems. Compared with the analysis of different mutants, the use of ubiquitous promoters equalizes the expression patterns among the genes studied and, therefore, allows a direct comparison of the phenotypic effects caused by the coding sequences. For example, differential sensitivity of the cytokinin receptors AKH4 and AHK3 to biologically active cytokinins has been described (Yonekura-Sakakibara *et al.*, 2004; Spíchal *et al.*, 2004; Romanov *et al.*, 2006), opening the possibility of controlling the sensitivity to the available active cytokinins by regulating the expression pattern of the receptors. As the relative abundance of each receptor varies in different tissues, this possibility might be realized *in planta*. While overexpression of various type-A *ARR*s confirmed their negative role with respect to signalling activity (Fig. 1B; Hwang and Sheen, 2001; Osakabe *et al.*,

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2002; Kiba *et al.*, 2003; Lee *et al.*, 2007; To *et al.*, 2007; Ren *et al.*, 2009), differences in phenotypes were also recorded. For example, overexpression of *ARR5* affected lateral root formation, whereas the closely related *ARR6* had little effect. Instead, it interfered with elongation of the primary root, which was not significantly changed by *ARR5* (Ren *et al.*, 2009). *ARR4* caused hypersensitivity to red light, and specifically interacted with Phytochrome B (Sweere *et al.*, 2001). *ARR16* appears unique among type-A family members in mediating cytokinin's function in leaf senescence (Ren *et al.*, 2009). These phenotypical differences are probably caused by the variable C-terminal domains of the type-A ARRs (Fig. 2). As cytokinin-dependent protein stability has been reported to be differentially affecting different members (To and Kieber, 2008; Ren *et al.*, 2009), this mechanism may further contribute to the different phenotypes observed. Overexpression experiments with type-B *ARR*s resulted in increased sensitivity to cytokinin, consistent with their role as transcriptional activators in the signalling cascade (Fig. 1B; Hwang and Sheen, 2001; Sakai *et al.*, 2001; Imamura *et al.*, 2003; Tajima *et al.*, 2004). In addition, different type-B *ARR*s caused differences in phenotypes, both in the tissues affected and in the severity of the abnormalities (Imamura *et al.*, 2003; Hass *et al.*, 2004; Tajima *et al.*, 2004; Choi *et al.*, 2010). Since little difference was observed in the DNA-binding affinity of the different B-type ARRs *in vitro* (Sakai *et al.*, 2000; Hosoda *et al.*, 2002; Imamura *et al.*, 2003), and different B-type ARRs could all mediate transcriptional activation of a synthetic cytokinin reporter *in vivo* (Müller and Sheen, 2008), the unique functions of different type-B ARRs are probably encoded by their variable C-terminal extensions. This was exemplified with ARR2 (Choi *et al.*, 2010), where the selection of cytokinin target genes is altered by the specific recruitment of DNA-binding cofactors. In summary, a number of gene-specific functions have been reported within families of the cytokinin signalling pathway, ranging from the receptors to the transcription factors. In cases where specificity is encoded by the protein sequences, the causative determinants in the protein sequence map to the variable amino acid residues of the different protein families, which mediate interaction with specific partners (Choi *et al.*, 2010), or exhibit altered biochemical activities (Mähönen *et al.*, 2006*a*, *b*). *In vitro,* the potential for differential protein interactions of cytokinin signalling genes has been uncovered by systematic screens (Dortay *et al.*, 2006, 2008). We are still far from a comprehensive view of how the individual protein signature of cytokinin signalling components contributes to signalling specificity. However, the identified specific functions provide a sound foundation to refine our understanding in a stepwise way. The contribution of differential expression to signalling specificity will be discussed further in the following sections.

The expression level of signalling components affects the pathway activity

At least one member of a hybrid kinase, *AHP* and B-type *ARR* must be expressed beyond a minimal threshold level for a cell to activate the signalling pathway. Increasing the expression levels of these positively acting components amplifies the signalling activity, while expression of the negatively acting type-A ARR and AHP6 attenuates it (Fig. 3). Therefore, the efficiency of phosphotransfer correlates with the expression levels of signalling components, which represents a potential mechanism as to how to tailor the sensitivity to a given stimulus. Tissue-specificity, feedback regulation from cytokinin signalling and other signalling inputs are responsible for the spatio-temporal expression profiles of the signalling components. Feedback regulation has been documented for the expression of type-A *ARR*s and *AHP6*. Type-A *ARR*s represent immediate-early transcriptional target genes that negatively interfere with signalling activity. Such a negative feedback loop may serve to smooth-out fluctuations in signalling or to shut off pathway activity more abruptly after transient stimuli. Like type-A ARRs, AHP6 suppresses pathway activity. However, its transcription is repressed by cytokinin signalling (Mähönen *et al.*, 2006*a*). This probably serves to generate sharper boundaries between signalling and non-signalling domains in a tissue.

Spatiotemporal expression map of signalling components defines a sensitivity landscape

In addition to feedback regulation, the complex expression patterns of hybrid kinases, *AHP*s, and *ARR*s suggest elaborate transcription regulation, which integrates tissue-specific, and multiple signalling inputs. Thus, a picture emerges where expression of cytokinin signalling components predefines a spatiotemporal map of signalling sensitivity. This allows localized signal amplification and attenuation, depending on the functional requirements.

Recent studies of cytokinin function at the cellular resolution have opened the door to study the functional relevance as to how cytokinin output is influenced by the expression profiles of signalling genes (Fig. 4; Leibfried *et al.*, 2005; Müller and Sheen, 2008; Gordon *et al.*, 2009; Moubayidin *et al.*, 2010; Zhao *et al.*, 2010). While knowledge of all present signalling genes in a given cell defines its sensitivity and competence, the actual signalling response ultimately depends on the local concentration of active cytokinins, which is difficult to determine, due to the small size of active ligands and their diversity. Thus, the key to analysing cytokinin's function in the context of the embryonic root-meristem specification was the construction of a synthetic promoter, which does not rely on secondary signalling or tissue-specific information. Rather, its activity reflects the cytokinin signalling output in the nucleus, that is, transcriptional activation mediated by type-B ARRs (Müller and Sheen, 2008). Reporter activity was observed in the hypophysis, precursor of the root stem cell system. Upon asymmetric cell division of the hypophysis, the resulting basal cell lineage down-regulated cytokinin output, while the apical cell maintained activity. This differential signalling output, which is essential for the development of the nascent root meristem, depends on auxin to activate the

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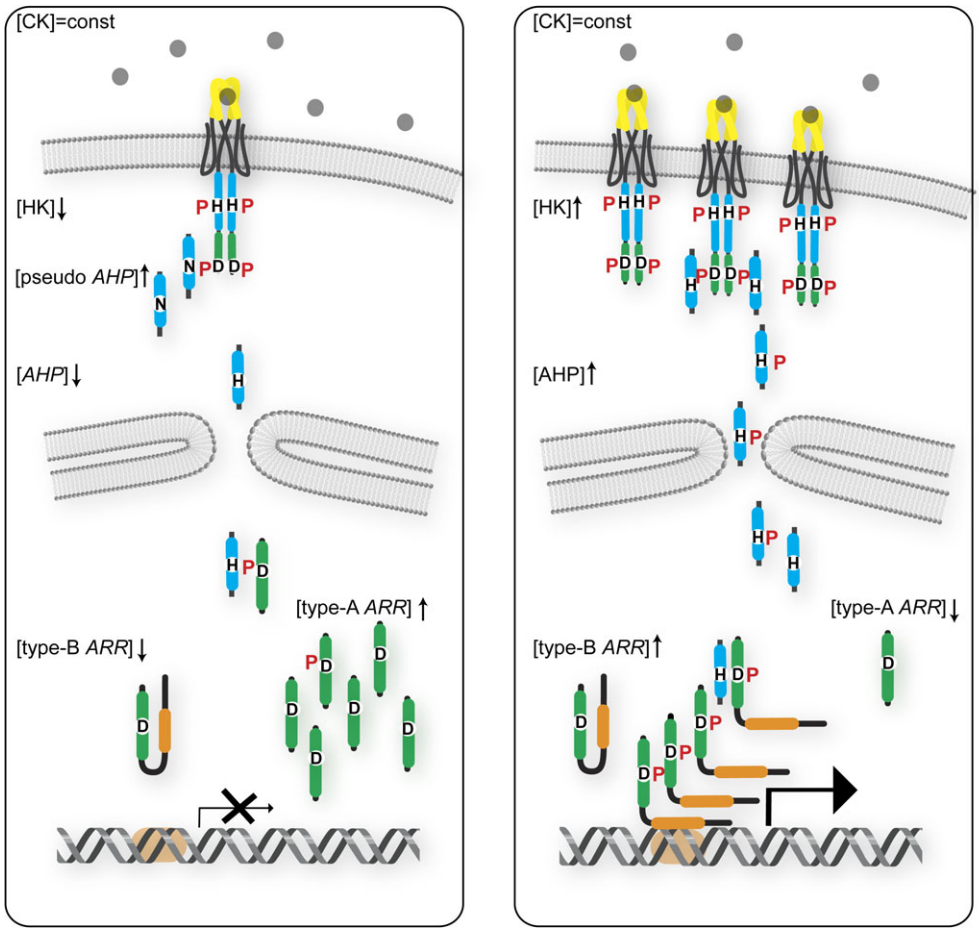

Fig. 3. Cartoon to illustrate how different expression levels of intracellular cytokinin signalling components affects the signalling activity, given a constant concentration of active ligands. On the left, signalling output is blocked due to prevalence of negative regulators: pseudo AHP6 and type-A ARRs. On the right, signalling output is maximized due to up-regulation of positive-acting signalling components: HKs, AHPs, type-B ARRs. Cytokinins are indicated as grey circles. Up-facing arrows indicate high concentrations, down-facing arrows low concentrations. Conserved domains are depicted as in Fig. 2.

transcription of the type-A *ARR7* and *ARR15* directly (Fig. 4C). Auxin signalling thus interferes with cytokinin signalling in a defined domain by inducing negative regulators, which suppresses the efficiency of phosphorelay. An analogous mechanism operates in the shoot apical meristem (Fig. 4D). However, interestingly, the relationship is reversed: here, auxin represses the transcription of *ARR7* and *ARR15*, leading to an increase in cytokinin signalling activity. Again, this cross-talk appears functionally relevant as interfering with *ARR7* and *ARR15* levels causes irregularities in phyllotaxis, the ordered arrangement of lateral shoots (Zhao *et al.*, 2010). In addition, WUS directly attenuates transcription of type-A *ARR5, ARR6, ARR7*, and *ARR15* (Leibfried *et al.*, 2005), again enhancing cytokinin-signalling activity. In the same context, an increase in receptor levels further contributes to increased perception in a spatially defined domain (Gordon *et al.*, 2009). Therefore, in the shoot apical meristem, both a decrease in the abundance of negative regulators (Leibfried *et al.*, 2005; Zhao *et al.*, 2010), and an increase in expression of a cytokinin receptor (Gordon *et al.*, 2009), a positive regulator, contributes to enhance the sensitivity to cytokinins. Cytokinin signalling in the transition zone of the adult root meristem counteracts proliferation by promoting differentiation (Dello Ioio *et al.*, 2007). Thus, exact signalling levels are critical for establishing the balance between growth and differentiation. During the early phases, when net growth of the root is required, the proliferation-inducing phytohormone Gibberellin selectively represses the transcription of type-B *ARR1*, hereby lowering the sensitivity to available cytokinins and hence signalling levels (Fig. 4E). At the later stages, when Gibberellin levels decrease, *ARR1* transcription and, consequently, cytokinin signalling increases, which tips the balance towards differentiation (Moubayidin *et al.*, 2010). Here, competence to respond to cytokinin is regulated by controlling the expression of a type-B *ARR*. These studies have revealed the functional importance of modulating pathway activity by controlling the expression levels of signalling components.

More detailed studies will probably follow to demonstrate how other signalling pathways determine the sensitivity landscape by amplifying or attenuating the cytokinin response. During female gametophyte development, cytokinin-signalling levels are controlled by the hybrid kinase

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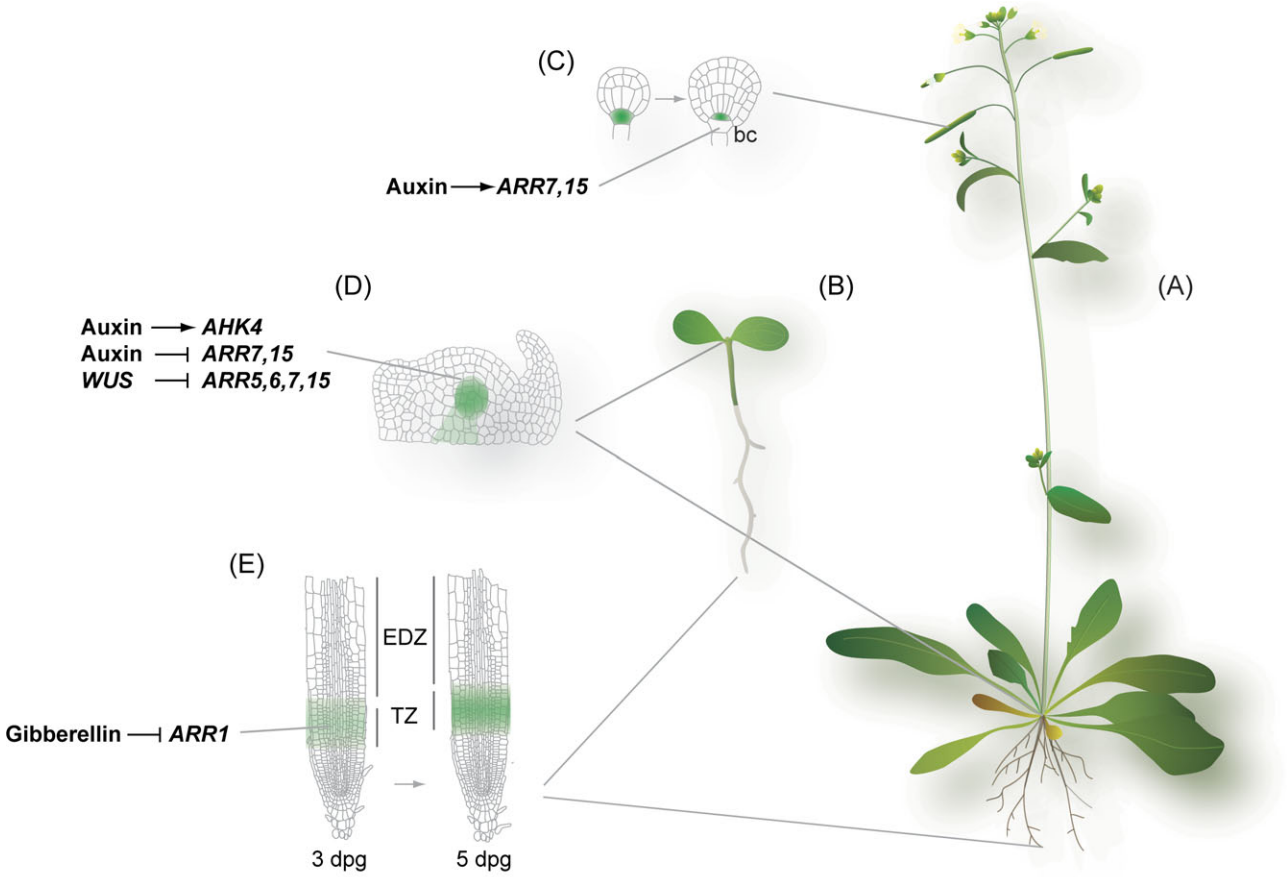

Fig. 4. The regulation of cytokinin signalling components affects the signalling output in different plant organs. Cartoons of an adult *Arabidopsis* plant (A), and a seedling (B). Schematic views of early embryos at the globular and transition stage (C), the shoot apical meristem (D), and the primary root (E) are drawn on the left. Cytokinin output is indicated in green, based on the expression of the synthetic TCS reporter in (C) (Müller and Sheen, 2008) and (D) (Zhao *et al.*, 2010), or inferred from the expression patterns of *ARR1* and *ARR12* and phenotypic analysis of the corresponding mutants (Moubayidin *et al.*, 2010) in (D). (C) Down-regulation of cytokinin output in the basal cell (bc) of a transition-stage embryo is caused by auxin, which induces the transcription of *ARR7* and *ARR15* (Müller and Sheen, 2008). In the shoot meristem (D), several mechanisms operate in parallel to up-regulate cytokinin sensitivity: auxin stimulates expression of *AHK4* (Gordon *et al.*, 2009), and represses transcription of *ARR7* and *ARR15* (Zhao *et al.*, 2010), while WUS represses transcription of *ARR5, ARR6, ARR7*, and *ARR15* (Leibfried *et al.*, 2005). In the primary root (E), Gibberellin signalling represses *ARR1* expression at the early stages (3 days post germination, dpg). Later, around 5 dpg, the mechanism is released, allowing expression of *ARR1* and increased cytokinin signalling output (Moubayidin *et al.*, 2010). TZ, transition zone; EDZ, elongation and differentiation zone. Refer also to the body text for further explanations.

CKI1 (Pischke *et al.*, 2002; Hejátko *et al.*, 2003), independent of the bona fide cytokinin receptors *AHK2, AHK3*, and *AHK4* (Deng *et al.*, 2010). CKI1 activates cytokinin signalling independent of cytokinins (Hwang and Sheen, 2001; Yamada *et al.*, 2001; Hejátko *et al.*, 2009; Deng *et al.*, 2010). Accordingly, pathway activity depends on the transcriptional profile of *CKI1*, and it will be interesting to identify the signals responsible. One candidate signal is auxin, which has been reported to be in involved in patterning of the embryo sac (Pagnussat *et al.*, 2009). By inducing *CKI1* expression, these signals can directly activate the cytokinin-signalling pathway, bypassing the need of cytokinins. Cytokinin's function in root gravitropism could, potentially, also be influenced by auxin (Aloni *et al.*, 2004). Transcription of the type-C ARR *ARR22* is induced by wounding (Gattolin *et al.*, 2006), which could reflect an additional case of how cytokinin output is determined by other signals. Recently, GeBP and GeBP-like transcription factors were identified that influence the cytokinin response by indirectly affecting the expression levels of type-A *ARR*s (Chevalier *et al.*, 2008). The concept of organizing cross-talk among hormone signalling pathways by controlling the expression levels of key signalling components is further exemplified by several well-documented cases where cytokinin signalling influences other signalling pathways (Laplaze *et al.*, 2007; Dello Ioio *et al.*, 2008; Ruzicka *et al.*, 2009).

The cellular response to cytokinin signalling qualitatively differs in the examples discussed above. In the nascent root meristem (Müller and Sheen, 2008) and during female gametophyte development (Pischke *et al.*, 2002; Hejátko *et al.*, 2003; Deng *et al.*, 2010), interfering with cytokinin function affects cell specification while, in the root,

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cytokinin has been shown to counteract auxin-promoted proliferation in the transition zone (Dello Ioio *et al.*, 2008). In the shoot meristem, cytokinin's role is to maintain the size and function of the stem-cell pool (To and Kieber, 2008; Gordon *et al.*, 2009; Werner and Schmülling, 2009; Zhao *et al.*, 2010). How can the same signalling pathway elicit these different responses? As discussed above, the specific set of cytokinin signalling proteins present in the cell may affect the signalling outcome. Along with the type-B ARR transcription factors, CYTOKININ RESPONSE FACTORS (CRF) have been reported to control a subset of immediate–early cytokinin target genes, further increasing the specificity of the signalling response (Rashotte *et al.*, 2006). In addition, the cellular status of the signal-receiving cell can affect how a signal is interpreted. In the case of cytokinin signalling, cytokinin-responsive promoters may rely on secondary regulatory input to confer transcriptional activation. For example, while transcription of type-A *ARR*s is sensitive to cytokinin, the transcription patterns only partly overlap, reflecting the dependence on additional input (To *et al.*, 2004; Leibfried *et al.*, 2005). Transcriptome changes in response to exogenous cytokinin treatment were characterized in different settings (Che *et al.*, 2002; Hoth *et al.*, 2003; Rashotte *et al.*, 2003; Brenner *et al.*, 2005). The overlap among cytokinin-target genes between the different experiments was relatively small, providing further arguments that, in addition to cytokinin signalling, tissue- and context-specific inputs are important. Transcription control of the *ARR7* and *ARR15* genes demonstrates how the cellular context influences promoter activity: the same *cis*-regulatory sequence integrates activating input by the auxin pathway in the embryonic root meristem (Müller and Sheen, 2008), and repressive auxin input in the shoot meristem (Zhao *et al.*, 2010). Future analyses aimed at characterizing the relevant cytokinin target genes during female gametophyte development, root meristem specification and maintenance, and shoot meristem homeostasis will provide the necessary basis to address further the question of target-gene specificity in well-defined developmental contexts at high resolution.

Concluding remarks

Signalling circuitries allow cells to change the gene expression profile specifically in response to cues originating from their outside. The limited number of signalling system contrasts with the numerous and diverse responses they elicit. On the one hand, gene-specific functions, both coded by *cis*-regulatory and coding sequence, contribute to specific responses. This also applies to the cytokinin signalling pathway—its relatively simple core pathway logic becomes increasingly complex with the multiple family members, each with a complex expression pattern and the potential to interact with diverse partners. On the other hand, the cellular context of signalling affects the cellular response triggered. Specifically, the integration of tissue-specific information and other signals by the *cis*-regulatory regions of potential target genes renders transcriptome changes context-dependent. Progress made in the last decade in the understanding of the cytokinin signalling pathway, and how it is specifically implemented in the developmental context, has been remarkable, and will continue at a fast pace. Recent studies (Mähönen *et al.*, 2006*a*; Dello Ioio *et al.*, 2008; Müller and Sheen, 2008; Gordon *et al.*, 2009; Hejátko *et al.*, 2009; Deng *et al.*, 2010; Moubayidin *et al.*, 2010; Zhao *et al.*, 2010) have demonstrated the importance of precisely tuning the levels of cytokinin signalling during development, which results in an elaborate spatiotemporal signalling landscape. The main focus here was on work using the reference plant *Arabidopsis*, however, increasing effort is being devoted to study cytokinin signalling in crop plants (Giulini *et al.*, 2004; Yonekura-Sakakibara *et al.*, 2004; Ito and Kurata, 2006) and plants with different life cycles, which allows the role of cytokinin in the evolution of the plant body plan to be studied (Pils and Heyl, 2009; Hellmann *et al.*, 2010).

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